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## Helix pomatia and Panulirus interruptus Hemocyanin

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## CHAPTER 7

### SUMMARIZING CONCLUSIONS

This thesis reports on the structure-function relationship of a molluscan and an arthropod hemocyanin.

#### *1. Length of the polypeptide chain of Helix pomatia $\alpha$ -hemocyanin (chapter 2)*

The high molecular weight of the polypeptide chain as determined with a variety of methods has led to a model with a number of covalently linked units. Electron microscopic studies (Siezen and van Bruggen 1974) did in fact show 1/20 molecules as chains of 7 or 8 globules, each with a diameter of 55-60 Å. This necklace structure seems to be a general feature of the molluscan hemocyanins (Lontie et al. 1973, Brouwer 1975, Gielens et al. 1975).

A number of intriguing questions still remain to be answered:

- (i) are the regions with a compact tertiary structure (structural domains) all similar regarding their structural and functional properties? For *Helix pomatia*  $\alpha$ -hemocyanin, Brouwer (1975) has shown that some of the domains are structurally very similar, but Gielens et al. (1975) showed that trypsinolysis of *Helix pomatia*  $\beta$ -hemocyanin resulted in fragments with markedly different properties as judged from CD spectroscopy.
- (ii) how are these domains arranged in the cylinder wall of the molecule? Brouwer (1975) proposed that two types of polypeptide chains occur in equimolar amounts in *Helix pomatia*  $\alpha$ -hemocyanin in order to explain the time course of the appearance of fragments due to proteolysis. In the case of *Helix pomatia*  $\beta$ -hemocyanin it is possible to remove the collar structure upon trypsinolysis, while only a few peptide bonds are hydrolyzed in the cylinder wall (van Breemen et al. 1975). This suggests a specific folding of the domains in the cylinder wall.

#### *2. Binding of carbon monoxide to $\alpha$ - and $\beta$ -hemocyanin from Helix pomatia (chapter 3)*

These hemocyanins bind carbon monoxide non-cooperatively, contrary to what has been observed for the binding of oxygen,

in which case a cooperative binding behaviour has been found under certain conditions (Konings et al. 1969). This difference in binding behaviour may reflect a different mode of ligand binding in the active center: carbon monoxide is bound with its oxygen atom to one of the copper atoms (Fager and Alben 1972), while oxygen is bound as a peroxide forming a non-planar Cu-O<sub>2</sub>-Cu complex (Freedman et al. 1976).

Heterotropic interactions (influence of hydrogen and calcium ions on the carbon monoxide equilibrium) are still present however. The fact that the cooperativity of the binding of ligand is not directly coupled to the release or uptake of H<sup>+</sup> or Ca<sup>2+</sup> ions by the protein suggests that the heterotropic interactions are due to local changes in the protein upon binding of the ligand. It is unknown whether the occurrence of heterotropic interactions is restricted to whole molecules.

Since the Bohr effect generated by the binding of carbon monoxide parallels the "oxygen Bohr effect", it is reasonable to suppose that in both cases the same groups are involved. However, the binding of carbon monoxide influences additional groups which are essential for stabilizing the quaternary structure of the molecule, since the protein dissociates into halves and tenths molecules at high pH in the presence of calcium ions upon binding carbon monoxide.

### 3. Structural and functional studies of *Panulirus interruptus* hemocyanin (chapter 4, 5 and 6)

Using several methods to determine molecular weights it was found that *Panulirus interruptus* hemocyanin is a hexamer. The molecule dissociates into monomers upon changes in hydrogen or calcium ion concentration; no species with molecular weights intermediate between hexamers and monomers were observed.

The dissociation behaviour of *Panulirus* hemocyanin indicates heterogeneity. Contrary to what has been observed for *Helix pomatia*  $\alpha$ -hemocyanin under conditions of moderate ionic strength, we did observe a certain concentration dependence for the composition of a mixture of hexamers and monomers, indicating that part of the molecules are in a dynamic equilibrium. This problem has been approached only qualitatively but, since we were able to fractionate the subunits into species with different chromatographic and electrophoretic properties, it may become possible to describe

the heterogeneity mo

Oxygen equilibrium of hemocyanin to bind oxygen under different conditions, while displaying cooperative oxygen binding with a lower affinity than whole *Panulirus* hemocyanin. The influence of oxygen on the change the position of the Bohr effect. For instance, preliminary results suggest that calcium is preferred by the protein.

A kinetic analysis of hemocyanin is described.

Stopped-flow experiments show (i) the oxygen dissociation of the protein is a homogeneous process dependent upon the pH of the solution and (ii) the oxygen dissociation of the protein exhibits heterogeneity, which is lost in the dead time of the apparatus.

Temperature-jump experiments show undissociated cooperative relaxation of heterogeneous relaxation of the monomers as compared with the relaxation as observed under equilibrium conditions, which is reflected in a higher temperature dependence.

These introductory studies on *Panulirus* hemocyanin are compared with other hemocyanins. One question is whether the observed heterogeneity is a reflection of changes in the heterogeneity of the protein or if it is a property of the changes.

The last part of the study is the fractionation of *Panulirus* hemocyanin into three fractions: functional and structural, and protein carried out studies on differences probably due to

Since heterogeneity

the heterogeneity more quantitatively.

Oxygen equilibrium studies showed undissociated hemocyanin to bind oxygen cooperatively under certain conditions, while dissociated hemocyanin exhibited non-cooperative oxygen binding behaviour, with a much lower oxygen affinity than whole molecules. Hydrogen and calcium ions caused a decrease in oxygen affinity of undissociated protein. Panulirus hemocyanin may be a good system to study the influence of oxygen on the release or uptake of ions which change the position and shape of the oxygen-binding curve. For instance, preliminary experiments (unpublished) indicate that calcium is preferably bound to the deoxy state of the protein.

A kinetic analysis of oxygen binding by Panulirus hemocyanin is described in chapter 5.

Stopped-flow experiments showed that:

- (i) the oxygen dissociation from undissociated cooperative protein is a homogeneous process as far as observable. It is dependent upon the pH of the solution which is a reflection of the Bohr effect found in equilibrium studies.
- (ii) the oxygen dissociation from dissociated hemocyanin exhibits heterogeneity with a high percentage of the reaction lost in the dead time of the apparatus.

Temperature-jump experiments showed for both the undissociated cooperative protein and the monomers a heterogeneous relaxation behaviour. The lower oxygen affinity of the monomers as compared to that of undissociated hemocyanin, as observed under equilibrium conditions, is kinetically reflected in a higher dissociation rate constant of the monomers.

These introductory studies on the kinetic behaviour of Panulirus hemocyanin show some striking differences with other hemocyanins. One of the main problems to be attacked is whether the observed heterogeneity in kinetic behaviour is a reflection of chromatographic and electrophoretic heterogeneity of the protein (as described in chapter 6), or is a property of the protein for instance due to conformational changes.

The last part of this thesis deals with heterogeneity of Panulirus hemocyanin. The dissociated hemocyanin may be fractionated into three components. Investigations of the functional and structural properties of the fractionated protein carried out so far, indicate that minor structural differences probably cause the observed heterogeneity.

Since heterogeneity seems to be a general feature of

arthropod hemocyanins (Sullivan et al. 1974, Murray and Jeffrey 1974, Sugita and Sekiguchi 1975) it may be rewarding to pay attention to such questions as:

- (i) what are the structural differences underlying the observed heterogeneity and
- (ii) how are these differences related to the functional behaviour of these hemocyanins.

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### SAMENVATTING

In dit proefschrift wordt de structuur van hemocyanine Hemocyanin functie het ver weekdieren en g de zuurstofspan hemocyaninemole relatief lage z gegeven. De zuu waarvan er twee

Een aantal sinds geruime t gemaakt:

(i) Hemocyanines van 450.000 tot mee hemoglobine, mens, met een m een zuurstofbind gewicht heeft v waarin het voork moleculen met e (Hemoglobine bez omstandigheden, ionenconcentrati aantal deeltjes, die ook nog in s

De electron hemocyaninemolec bij weekdieren, en moleculen met (voorkomend bij *Panulirus interruptus* duidelijk waar t

is afgedrukt. (ii) Hemocyanines gedrag. In Figuur verzadigingsgraac zuurstofspanning